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A Rapid and Reliable Procedure for Extraction of Cellular Polyamines and Inorganic Ions from Plant Tissues*

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Abstract. A fast and reliable method for the extraction of cellular polyamines and major inorganic ions (Ca, Mg, Mn, K, and P) from several plant tissues is described. The method involves repeated freezing and thawing of samples instead of homogenization. The efficiency of extraction of both the polyamines and inorganic ions by these two methods was compared for 10 different tissues. In each case, the freeze–thaw procedure resulted in a precise and quantitatively equal, or greater, yield than homogenization. Freeze–thawing not only eliminates the need for various tissue homogenizers (such as polytrons, tissumizers, and mortars and pestles), but it is so simple that a large number of samples can be processed simultaneously. We routinely processed 50–80 samples for quantitation of polyamines and inorganic ions. Freeze–thawing was equally useful for the extraction of polyamines from liver, spleen, and kidney tissues of mice.

Polyamines, spermidine, spermine, and their precursor, putrescine, are simple, open-chained, polybasic carbon compounds. Polyamines play an important role in the growth and development of plants (Flores et al. 1990, Slocum and Flores 1991). This information, along with their ubiquitous occurrence, has prompted numerous studies on the quantitative determination of cellular levels of polyamines in various plant tissues (Minocha et al. 1990, Smith 1991).

It has been suggested that divalent inorganic cations such as Ca and Mg can substitute for polyamines in some metabolic activities, especially under stress conditions (Smith 1985, Minocha et al. 1992). As a result, there is a need to analyze cellular levels of both the polyamines and inorganic cations from the same tissues.

Most of the published work on extraction of polyamines from various plant tissues involves homogenization in cold perchloric acid (PCA) or trichloroacetic acid (TCA) using either one of the following: a) chilled mortar and pestle with liquid nitrogen, b) polytron or tissumizer, or c) small conical ground-glass homogenizers with matching pestles (Birecka et al. 1988, Faure et al. 1991, Kushad and Yelenosky 1987, Maki et al. 1991, Meijer and Simmonds 1988, Minocha et al. 1991, 1992, 1993, Nielsen 1990, Rastogi and Davies 1989, Torrigiani et al. 1987). Even though any of these extraction procedures is not too complicated when relatively few samples need to be processed, they are definitely time consuming and occasionally noisy due to the use of polytron for longer periods. Above all, these methods are less precise, especially when the sample size is very small. Each time a probe or a pestle is used to homogenize a sample, the amount of tissue lost may be different due to operator error and/or precautions taken to clean the probe between samples. This sample loss is even more significant when dealing with fibrous tissues such as leaves and needles or root material from trees. These tissues tend to get entangled in the probe of the tissumizer.

Wet acid digestion at high temperature is the most widely used method for determination of total inorganic ions from various plant tissues. Recently, new microwave digestion methods have also been used for this purpose (Kingston and Jassie 1986). Most of these procedures require a large sample size (100–1000 mg) and are also time consuming, laborious, costly, and, in some cases, hazardous (Anderson and Henderson 1986, Isaac and Johnson

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1976, Kingston and Jassie 1986, Kuennen et al. 1982, Wikoff and Moraghan 1986, Wolf 1982). These methods are often used for diagnostic crop nutrient status. A faster and safer procedure that is suitable for small sample sizes would be useful to various laboratories for quantification of either total or exchangeable levels of major inorganic ions.

In this paper, we describe a rapid and reliable procedure for the extraction of polyamines and major inorganic ions from various plant tissues. Combined with the method of HPLC described earlier (Minocha et al. 1990), up to 100 samples can be analyzed for polyamines within a 24-h period.

Materials and Methods

Tissue Preparation

In the case of whole plants, leaves or needles from one or more plants were pooled, washed with distilled water, blot dried, and finely chopped. Callus tissue grown on agar-solidified medium was pooled from several plates, blotted on filter paper to remove excess moisture, and finely chopped before weighing. Two or more flasks containing cells growing in suspension culture were mixed, collected on Miracloth (Calbiochem-Behring Corp., La-Jolla, CA, USA), and thoroughly washed with three volumes of deionized distilled water. Aliquots were taken from these pooled tissues for comparison of different methods as well as for replicates within the same method. The tissues tested were callus of Norway spruce (*Picea abies* L. [Karst]), tobacco (*Nicotiana tabaccum*), and aspen (*Populus tremuloides*); cell suspensions of red spruce (*Picea rubens* [Sarg.]), carrot (*Daucus carota*), hybrid poplar (*Populus nigra* × *maximowiczii*), and periwinkle (*Catharanthus roseus*); leaves of carrot and needles of red spruce seedlings grown in the greenhouses (1 year old, 2 years old, or mixed needles); and roots of 2-year-old red spruce seedlings. Tissue samples were replicated 4–5 times unless stated otherwise.

The growth conditions for different tissues are described as follows: Norway spruce and red spruce (Minocha et al. 1993), tobacco (DeScenzo and Minocha 1993), carrot (Robie and Minocha 1989), periwinkle (Minocha et al. 1992), aspen (Noh and Minocha 1990), and hybrid poplar (Sun et al. 1994). Liver, spleen, and kidney tissues from 2–3 mice were finely chopped and tested as representative samples of animal tissues for extraction of polyamines.

Extraction by Homogenization

For the extraction of inorganic ions by homogenization, 5 ml of 0.01N HCl were added to a 50-mg tissue sample in a 15-ml acid-washed glass test tube. The samples were homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer (Brinkmann Instruments, Inc., Westbury, NY, USA). The extracts were stored in test tubes at 4°C until the time of analysis. Samples were filtered by using a 45-μm nylon syringe filter immediately before analysis.

For extraction of soluble polyamines, 200-mg tissue samples were transferred to 5% PCA (tissue:PCA ratio 1:4 w/v) in a 15-ml Corex centrifuge tube and homogenized for 90 s at 20,000 rpm using a Brinkmann Polytron homogenizer. The samples were incubated on ice for 1 h, centrifuged at 18,000*g* for 20 min, and the supernatant kept frozen at −20°C until needed.

Extraction by Freeze–Thawing

For freeze–thaw extraction, the sample weight, extraction solvents, and extraction volume were kept identical to the homogenization method except that the samples for polyamines were processed in 1.5-ml microfuge tubes. The samples for both inorganic ions as well as for polyamines were frozen at −20°C and thawed at room temperature, repeating the process two more times. Duration of the freezing step could vary from 4 h to a few days. Samples were allowed to thaw completely (approximate time, 1–1.5 h) before refreezing. After freeze–thawing, the samples were either filtered (for inorganic ions) or centrifuged at 13,500*g* (for polyamines). For extraction of polyamines from small size samples (25–200 mg), the same ratio (1:4) of tissue to PCA was used.

Ion Analysis

Concentrations of major inorganic ions, Ca, K, Mg, Mn, and P, were determined by Beckman Spectrospan V ARL DCP-AES (Direct Current Plasma Atomic Emission Spectrometer, Beckman Instruments Inc., Fullerton, CA, USA) using the Environmental Protection Agency's (EPA) method number 66-AES0029 (1986).

Dansylation and Quantification of Polyamines

Prior to dansylation, heptanediamine was added to the extracts as an internal standard for polyamine analysis. Fifty microliters of the extract was dansylated according to the procedure described in Minocha et al. (1990). Dansylated polyamines were separated by reversed-phase HPLC (Perkin-Elmer Corp., Norwalk, CT, USA), using a gradient of acetonitrile and heptanesulfonate, and quantified by a fluorescence detector (Minocha et al. 1990).

Statistical Analysis

Systat 5.02 (Systat Inc., Evanston, IL, USA, 1992) for Windows was used to perform either one-way analysis of variance or t test for independent samples to evaluate if the treatment means were significantly different (two-tailed, $\alpha = .05$). An f test for homogeneity of variance was performed on data to determine if a t test for equal or unequal variances should be used (Snedecor and Cochran 1956). In cases where the null hypothesis of homogeneity was accepted, pooled variances were used for the t test. In cases where the null hypothesis of homogeneity was rejected, t test for the separate variances was performed.

Results

Polyamines

Freeze–thawing resulted in extraction of equal or significantly higher levels of free putrescine as well as spermidine for all tissues tested (Fig. 1). Sper-

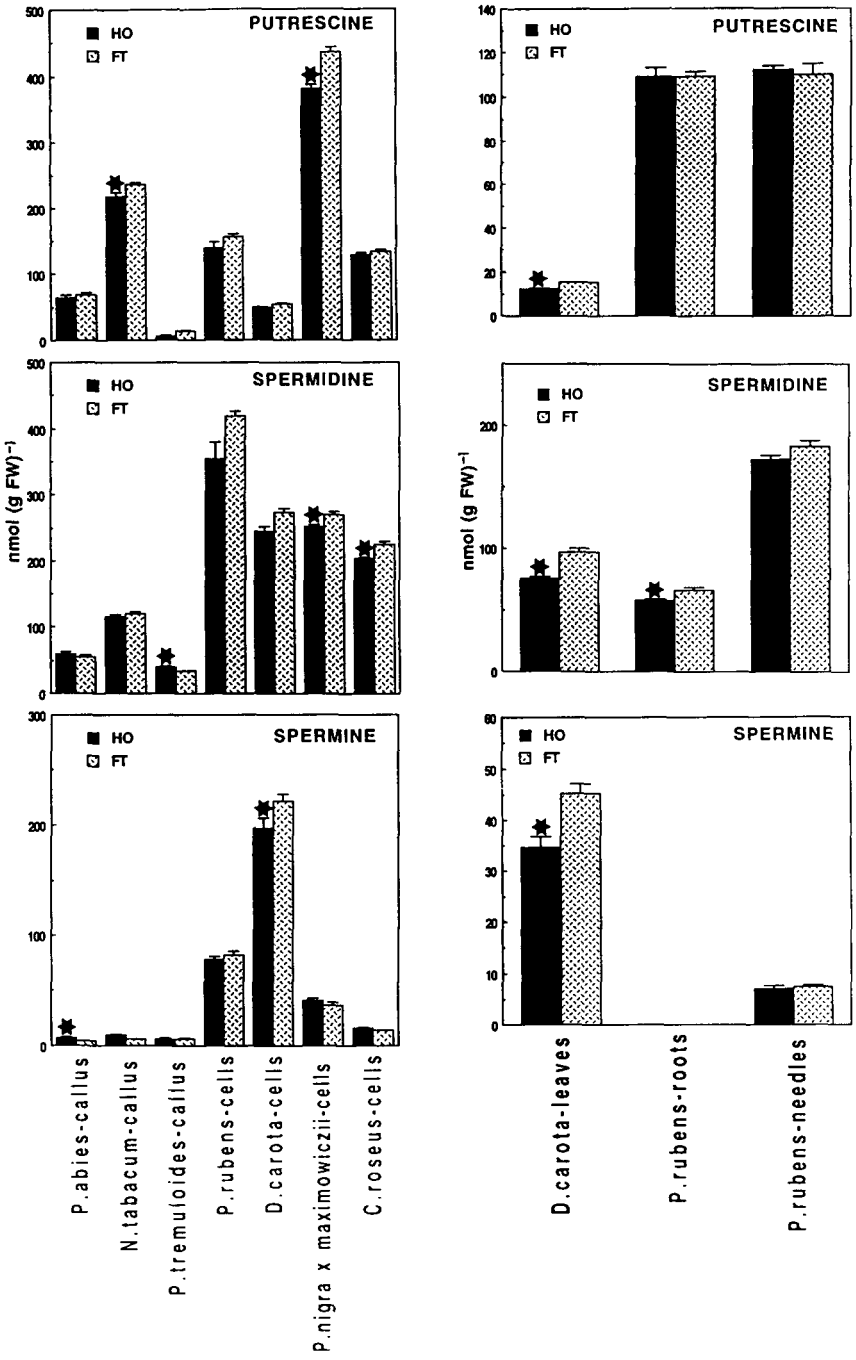

Fig. 1. Comparison of homogenization (HO) and freeze–thawing (FT) for extraction of putrescine, spermidine, and spermine from various tissues. Data are mean ± SE of 5 replicates. Asterisk indicates significant difference ($p \leq .05$) between homogenization and freeze–thawing.

mine was barely detectable in most tissues, except for red spruce cells and carrot, and could be extracted by freeze–thawing. For all plant tissues tested for extraction of polyamines, the precision for freeze–thawing was as high or higher than homogenization. There were no differences in the kinds of polyamines detected by either method for any of the tissues.

Once it was established that freeze–thawing could replace homogenization for extraction of free polyamines, we used hybrid poplar cells, carrot cells, and Norway spruce callus to test the freeze–thawing procedure for a range of small sample sizes (25–200 mg). The ratio of tissue to PCA (1:4) was maintained for each of the sample sizes tested. Results of this experiment indicate that 50 or 100 mg of all three tissues were optimum for the freeze-thaw extraction of polyamines in a 1.5-ml microfuge tube (Fig. 2). However, there was no significant difference in precision for any of the four sizes tested.

The freeze–thaw extraction technique yielded equal or significantly higher amounts of putrescine,

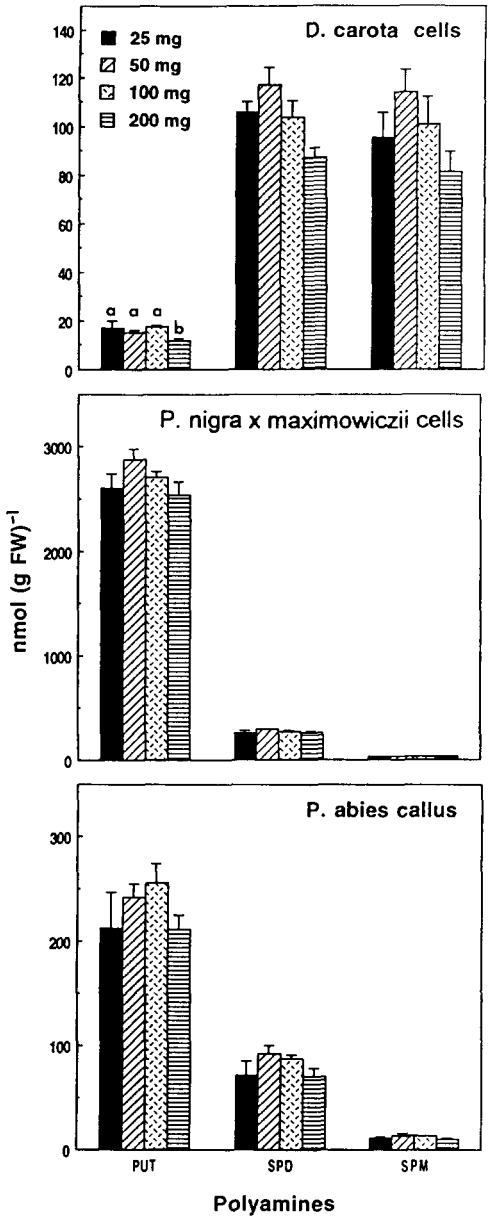

Fig. 2. Comparison of variable sample amounts for extraction of putrescine, spermidine, and spermine from three tissues using freeze–thaw extraction. Data are mean ± SE of five replicates. Different letters in top panel indicate significant differences between treatments.

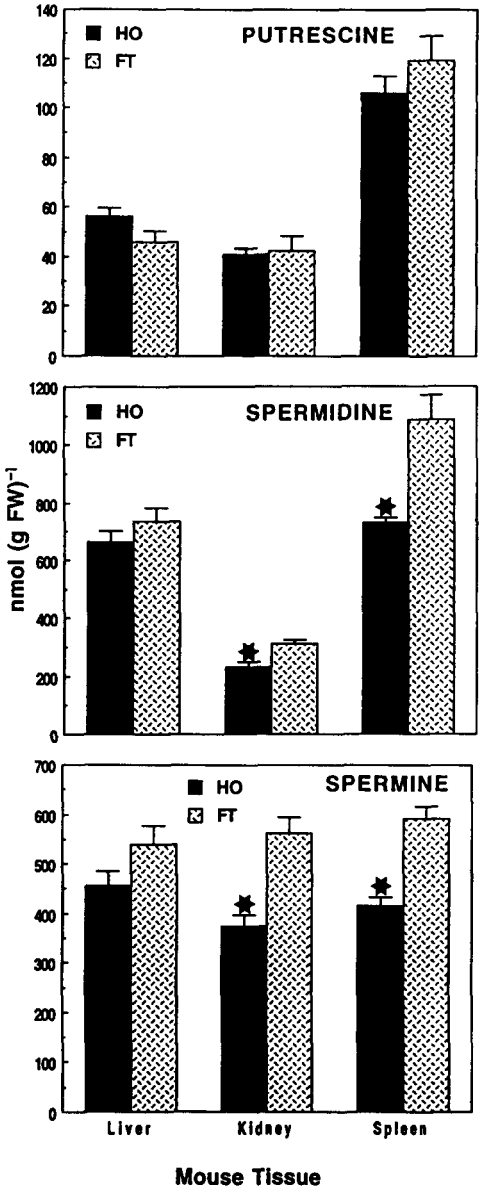

Fig. 3. Comparison of homogenization (HO) and freeze–thawing (FT) for extraction of putrescine, spermidine, and spermine from different mouse tissues. Data are mean ± SE of five replicates for liver tissue and three replicates each for kidney and spleen tissue. Asterisk indicates significant difference ($p \leq .05$) between homogenization and freeze–thawing.

spermidine, and spermine for the mouse tissues (Fig. 3). Unlike plant tissues, mouse tissues had spermidine and spermine as the major polyamines, whereas putrescine was present in small quantities.

Inorganic Ions

The freeze–thawing method extracted equal or significantly higher amounts of Ca, Mg, Mn, K, and P than homogenization from Norway spruce callus, tobacco callus, carrot cell suspensions, hybrid poplar cell suspensions, red spruce roots and needles (Figs. 4 and 5). The only exception was P extraction from carrot cell suspensions. For most other tissues tested P extraction by freeze–thawing was either similar to or significantly higher than homogenization, whereas for carrot cells it was significantly lower (Fig. 5).

The freeze–thaw extraction yielded slightly lower but not significantly different amounts of Ca, Mg,

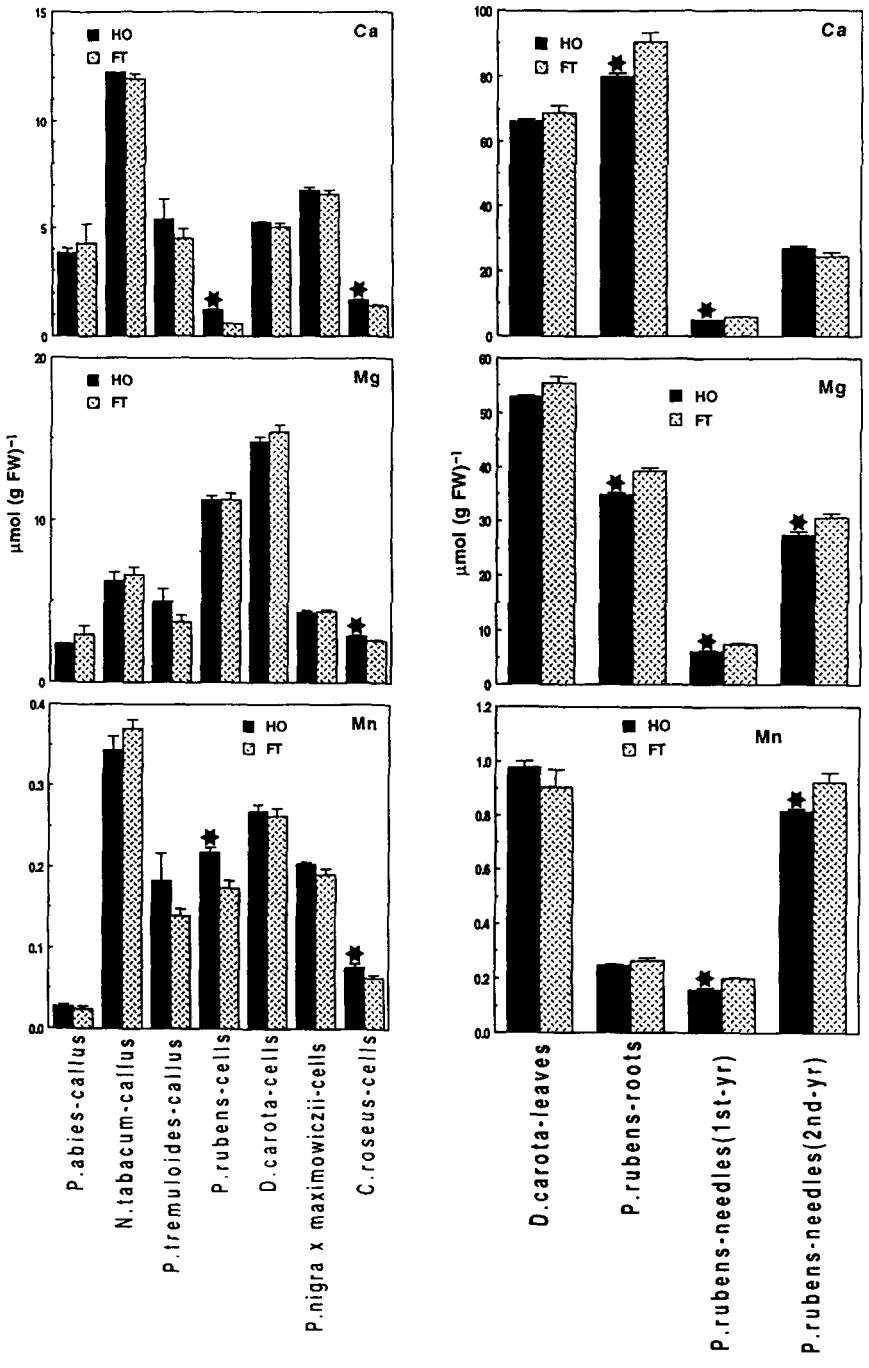

Fig. 4. Comparison of homogenization (HO) and freeze–thawing (FT) for extraction of Ca, Mg, and MN from various tissues. Data are mean ± SE of four replicates for homogenization and five replicates for freeze–thawing. Asterisk indicates significant difference ($p \leq .05$) between homogenization and freeze–thawing.

Mn, K, and P for aspen callus (Figs. 4 and 5). The higher degree of variation between replicates of this tissue for homogenization may be a reflection of the tissue heterogeneity.

In the case of *C. roseus* and red spruce cells, both Ca and Mn yields were significantly lower for freeze–thaw extraction than for homogenization. However, there was always a high degree of precision for freeze–thaw extraction as determined by low standard error bars for replicates (Figs. 4 and 5).

Discussion

Homogenization of samples is a relatively slow extraction procedure when considering that each sample must be processed separately. In contrast, by using freeze–thaw extraction one can process hundreds of samples per day. Freeze–thawing eliminates the need for tissue homogenizers for extraction of soluble polyamines. In most cases, the freeze–thawing procedure resulted in slightly higher values for polyamines. This may be due to the loss

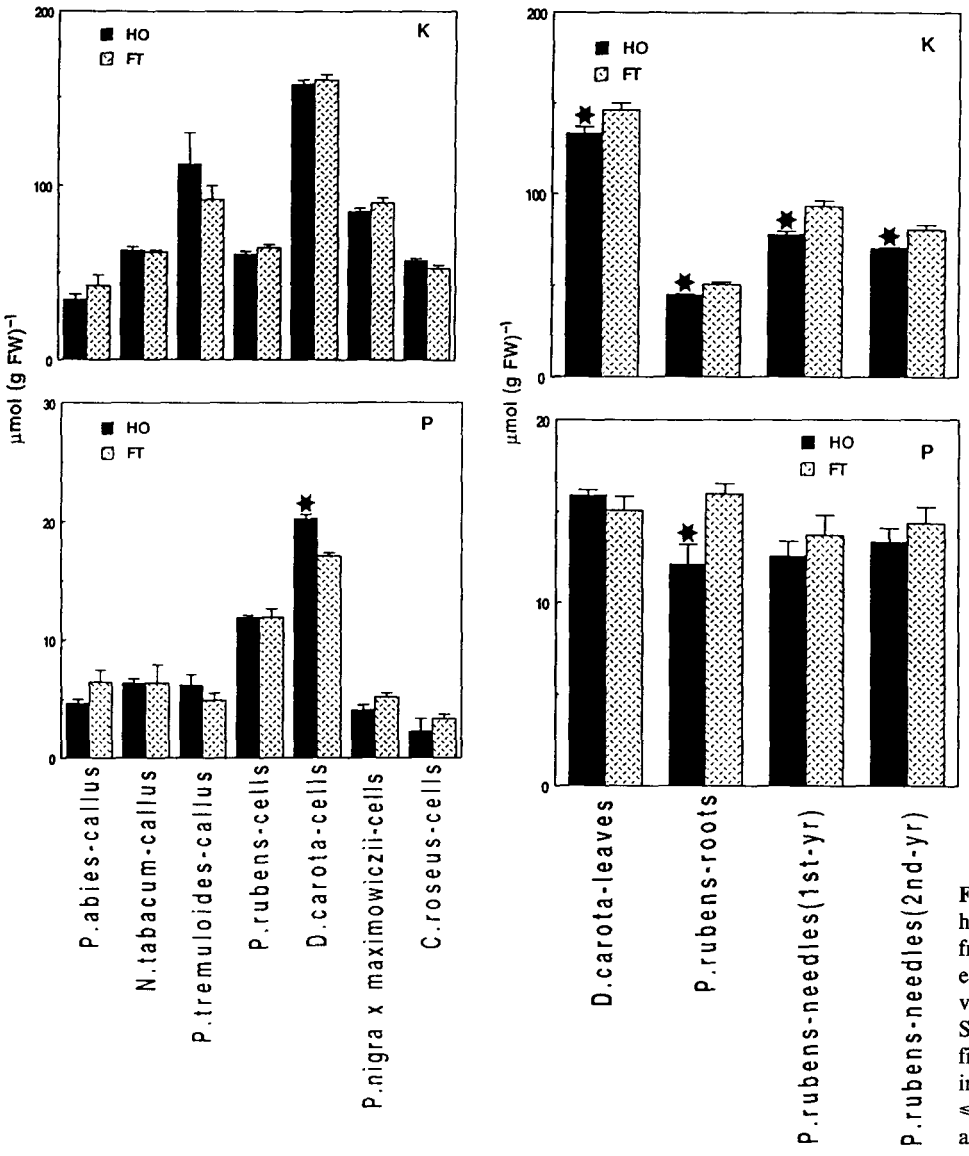

Fig. 5. Comparison of homogenization (HO) and freeze–thawing (FT) for extraction of K and P from various tissues. Data are mean ± SE of four replicates for HO and five replicates for FT. Asterisk indicates significant difference ($p \leq .05$) between homogenization and freeze–thawing.

of a small fraction of sample sticking to the probe of the polytron during homogenization. Thus, in our opinion, freeze–thawing provides a more accurate measure of the values for PCA-soluble polyamines in these samples.

The yields of major inorganic ions, although comparable between the two methods for most of the tissues tested, may or may not represent total ions present in these tissues. It is expected that both these procedures would extract only freely exchangeable or soluble ions from the tissue. Total ions from herbaceous leafy or nonleafy samples obtained from fresh or dry plant tissues are generally extracted by either wet acid or dry ashing digestions. However, the portion extracted by homogenization or freeze–thawing in our studies is always consistent and precise, as indicated by very small standard error bars. In the case of needles from 1-year-old red spruce seedlings, each of these ions were completely extracted by either homogenization, freeze–thawing, or wet acid digestion (Minocha and Shortle 1993). Thus, if the aim is to observe changes in freely exchangeable portion of inorganic ions in relation to a particular stress condition or a change in physiological state of the tissue, freeze–thawing may be appropriate to use. Another major advantage of replacing wet acid or dry ashing with freeze–thawing or homogenization is laboratory safety. It will eliminate the need for special equipment (digestion block or pressure bomb), large dilutions, concentrated acids, and large sample size.

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References

Anderson DL, Henderson LJ (1986) Sealed chamber digestion for plant nutrient analysis. Agron J 78:937–939

Birecka H, Birecki M, Cohen EJ, Bitonti AJ, McCann PP (1988) Ornithine decarboxylase, polyamines, and pyrrolizidine alkaloids in *Senecio* and *Crotalaria*. Plant Physiol 86:224–230

DeScenzo RA, Minocha SC (1993) Modulation of cellular polyamines in tobacco by transfer and expression of mouse ornithine decarboxylase cDNA. Plant Mol Biol 22:113–127

Faure O, Mengoli M, Nougarede A, Bagni N (1991) Polyamine pattern and biosynthesis in zygotic and somatic embryo stages of *Vitis vinifera*. J Plant Physiol 138:545–549

Flores HE, Arteca RN, Shannon JC (1990) Polyamines and ethylene: Biochemistry, physiology and interactions. American Society of Plant Physiologists, pp 1–425

Isaac RA, Johnson WC (1976) Determination of total nitrogen in plant tissue using a block digestor. J AOAC 59:98–100

Kingston HM, Jassie LB (1986) Microwave energy for acid decomposition at elevated temperatures and pressures using biological and botanical samples. Anal Chem 58:2534–2541

Kuennen RW, Wolnik KA, Fricke FL (1982) Pressure dissolution and real sample matrix calibration for multielement analysis of raw agricultural crops by inductively coupled plasma atomic emission spectrometry. Anal Chem 54: 2146–2150

Kushad MM, Yelenosky G (1987) Evaluation of polyamine and proline levels during low temperature acclimation of citrus. Plant Physiol 84:692–695

Maki H, Ando S, Kodama J, Komamine A (1991) Polyamines and the cell cycle of *Catharanthus roseus* cells in culture. Plant Physiol 96:801–806

Meijer EGM, Simmonds J (1988) Polyamine levels in relation to growth and somatic embryogenesis in tissue cultures of *Medicago sativa* L. (alfalfa). J Exp Bot 39:787–794

Minocha R, Kvaalen H, Minocha SC, Long SL (1993) Polyamines in embryogenic cultures of Norway spruce (*Picea abies*) and red spruce (*Picea rubens*). Tree Physiol 13: 365–377

Minocha R, Minocha SC, Komamine A, Shortle WC (1991) Regulation of DNA synthesis and cell division by polyamines in *Catharanthus roseus* suspension cultures. Plant Cell Rep 10:126–130

Minocha R, Minocha SC, Long SL, Shortle WC (1992) Effects of aluminum on DNA synthesis, cellular polyamines, polyamine biosynthetic enzymes, and inorganic ions in cell suspension cultures of a woody plant, *Catharanthus roseus*. Physiol Plant 85:417–424

Minocha SC, Minocha R, Robie CA (1990) High-performance liquid chromatographic method for the determination of dansyl-polyamines. J Chromatogr 511:177–183

Minocha R, Shortle WC (1993) Fast, safe, and reliable methods for extraction of major inorganic cations from small quantities of woody plant tissues. Can J For Res 23:1645–1654

Nielsen KA (1990) Polyamine content in relation to embryo growth and dedifferentiation in barley (*Hordeum vulgare* L.). J Exp Bot 41:849–854

Noh EW, Minocha SC (1990) Pigment and isozyme variation in aspen shoots regenerated from callus culture. Plant Cell Tiss Org Cult 23:39–44

Rastogi R, Davies PJ (1989) Polyamine metabolism in ripening tomato fruit. Plant Physiol 94:1449–1455

Robie CA, Minocha SC (1989) Polyamines and somatic embryogenesis. I. The effects of difluoromethylornithine and difluoromethylarginine. Plant Sci 65:45–54

Slocum RD, Flores HE (1991) Biochemistry and physiology of polyamines in plants. CRC Press, Boca Raton

Smith TA (1985) Polyamines. Ann Rev Plant Physiol 36:117–143

Smith MA (1991) Chromatographic methods for the identification and quantitation of polyamines. In: Slocum RD, HE Flores (eds) Biochemistry and physiology of polyamines in plants. CRC Press, Boca Raton, pp 229–242

Snedecor GW, Cochran WG (1956) Statistical methods. The Iowa State University Press, Ames

Sun DY, Minocha R, Minocha SC (1994) Genetic transformation of *Populus* to modulate polyamine metabolism. In: Ahuja MR (ed) Proceedings of the IUFRO workshop on biotechnology of trees, Valsain, Spain, 18–22 October 1993. (in press)

Torrigiani P, Serafini-Fracassini D, Bagni N (1987) Polyamine biosynthesis and effect of dicyclohexylamine during the cell cycle of *Helianthus tuberosus* tuber. Plant Physiol 84:148–152

Wikoff LR, Moraghan JT (1986) Determination of plant iron, manganese and zinc by wet ashing procedures. J Sci Food Agric 37:839–844

Wolf B (1982) A comprehensive system of leaf analyses and its use for diagnostic crop nutrient status. Commun Soil Sci Plant Anal 13:1035–1059